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Splice variants and expression patterns of *SHEP1*, *BCAR3* and *NSP1*, a gene family involved in integrin and receptor tyrosine kinase signaling

Virginie S Vervoort[1,*], **Séverine Roselli**[1,*], **Robert G Oshima**[1,2], and **Elena B Pasquale**[1,2,3]

1 *The Burnham Institute for Medical Research, La Jolla, California 92037*

2 *Pathology Department, University of California San Diego, La Jolla, California 92093*

Abstract

SHEP1, BCAR3 and NSP1 are the three members of a family of cytoplasmic proteins involved in cell adhesion/migration and antiestrogen resistance. All three proteins contain an SH2 domain and an exchange factor-like domain that binds both Ras GTPases and the scaffolding protein Cas. *SHEP1*, *BCAR3* and *NSP1* mRNAs are widely expressed in tissues, and *SHEP1* and *BCAR3* have multiple splice variants that differ in their 5′ untranslated regions and in some cases the beginning of their coding regions. Interestingly, our data suggest that *SHEP1* is highly expressed in blood vessels in mouse breast cancer models. In contrast, *BCAR3* and *NSP1* are more highly expressed than *SHEP1* in breast cancer cells. These expression patterns suggest differential roles for the three genes during breast cancer progression in either the vasculature or the tumor cells.

Keywords

endothelial cell; vascular endothelial growth factor; tumor stroma; breast cancer

1. Introduction

Only three related proteins are known to contain both an SH2 domain and a domain similar to guanine nucleotide exchange factor domains for Ras family GTPases. They are SHEP1 (SH2 domain-containing Eph receptor-binding protein 1, also known as CHAT, and NSP3; gene name *SH2D3C*) (Dodelet et al., 1999;Lu et al., 1999;Sakakibara and Hattori, 2000), BCAR3 (breast cancer antiestrogen resistance locus 3, also known as AND-34, SHEP2, NSP2 and SH2D3B; gene name *BCAR3*) (van Agthoven et al., 1998;Cai et al., 1999;Dodelet et al., 1999;Lu et al., 1999), and NSP1 (novel SH2-containing protein 1, gene name *SH2D3A*) (Lu et al., 1999). SH2 domains and exchange factor domains for Ras family GTPases are typically found in different proteins and brought together through protein-protein interactions (Pawson and Scott, 1997). In SHEP1, BCAR3 and NSP1 these two domains are instead stably connected by a serine/proline-rich linker region.

[3]Correspondence: Elena B. Pasquale, The Burnham Institute for Medical Research, 10901 N. Torrey Pines Rd., La Jolla CA 92037, 858 646 3131 (telephone), 858 646 3199 (FAX), elenap@burnham.org
[*]These two authors contributed equally

Through the SH2 domain, SHEP1, BCAR3 and NSP1 bind to tyrosine phosphorylated motifs in activated receptor tyrosine kinases such as Eph receptors and ErbB receptors (Dodelet et al., 1999;Lu et al., 1999;Jones et al., 2006). The exchange factor-like domain binds certain Ras proteins, but seems to lack exchange activity (Dodelet et al., 1999;Bos et al., 2001;Sakakibara et al., 2002). In all three proteins this domain also binds the scaffolding protein Cas (Cai et al., 1999;Lu et al., 1999;Sakakibara and Hattori, 2000), which is a critical component of integrin adhesion complexes regulating cell proliferation and migration/invasion (Bouton et al., 2001). Therefore, SHEP1, BCAR3 and NSP1 are cytoplasmic signaling intermediates that may function as adaptors or scaffolding proteins to integrate growth factor receptor and integrin signals.

SHEP1 and BCAR3 have been implicated in the regulation of cell morphology, adhesion and migration (Sakakibara et al., 2002;Cai et al., 2003a;Cai et al., 2003b;Riggins et al., 2003;Dail et al., 2004). Overexpression of SHEP1 and NSP1 has been found to promote c-Jun N-terminal kinase (JNK) activation (Lu et al., 1999;Sakakibara and Hattori, 2000;Sakakibara et al., 2003), a function that may depend on association with Cas (Dolfi et al., 1998;Sakakibara and Hattori, 2000). Because JNK plays a role in cell survival, proliferation and migration/invasion (Dunn et al., 2002;Xia and Karin, 2004), overexpression of SHEP1 or NSP1 in cancer cells could lead to a more malignant phenotype. Furthermore, overexpression of BCAR3 enables proliferation of estrogen-dependent breast cancer cells in the presence of the anti-estrogen tamoxifen (van Agthoven et al., 1998). Thus, it is important to understand how the expression of SHEP1, BCAR3 and NSP1 is regulated in normal and cancer cells. Here we report that human and mouse *SHEP1* and *BCAR3* have multiple splice variants, and characterize their expression patterns. Furthermore, *NSP1* appears to be a functional gene in the human but not the mouse genome.

2. Materials and Methods

2.1. Database searches and sequence analysis

We obtained the sequences of the different splice variants of the human SHEP1, BCAR3 and NSP1 genes from the Ensembl website (www.ensembl.org) for *Homo sapiens*. The corresponding mouse sequences were obtained from the Ensembl website for *Mus musculus* as well as the literature (Dodelet et al., 1999) and BLAST searches of NCBI (National Center for Biotechnology Information) databases. The amino-terminal sequence we report for *SHEP1*β is different from that published by Lu et al. (Lu et al., 1999) but corresponds to the sequence submitted by the authors to GenBank.

2.2. Cell lines and tumor xenografts

The MDA-MB-435 (Noren et al., 2004), YS2 (Balconi et al., 2000), and PAE (Miyazono et al., 1987) cells have been described. Human umbilical vein endothelial (HUVE) cells were purchased from Clonetics BioWhittaker Inc. or Cascade Biologicals and the other cell lines from ATCC (Rockville). Each cell line was maintained in the culture medium recommended by the vendor or as described in the above references. The growth media were supplemented with penicillin and streptomycin. To generate tumor xenografts, 3×10^6 MDA-MB-231 human breast cancer cells in a 0.1 ml volume were injected into the mammary fat pad region of female Balb/C nude mice as previously described (Noren et al., 2004). Mice were sacrificed 8 weeks after injection to collect tumor tissue.

2.3. mRNA extraction and RT-PCR analysis

mRNA was extracted from cultured cells, mouse brain, and tumor xenograft tissue using the Micro-FastTrack™ 2.0 kit (Invitrogen) according to the manufacturer's recommendations. For

each sample, 1 μg of mRNA was reverse-transcribed into cDNA in a 40 μl reaction using random hexamers with the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems) and 1–3 μl of the resulting cDNA were amplified per PCR reaction. Primers were designed to hybridize to different exons (Suppl. Table S1). For analysis of PCR products amplified from MDA-MB-231 tumor xenografts, the *SHEP1* products were digested with the enzyme *Bgl*II, which specifically digests the mouse sequence, and the *BCAR3* products were digested with *Apa*LI, which specifically digests the human sequence.

2.4. PCR amplification of splice variants

Human *SHEP1*, *BCAR3*, and *NSP1* splice variants were amplified from the Human Multiple Tissue cDNA panel I (BD Biosciences Clontech). Mouse *SHEP1α*, *SHEP1β*, and *SHEP1γ* were amplified from the Mouse Multiple Tissue cDNA panel I. Primers were designed to hybridize to different exons (Suppl. Table S1). The intensity of the bands amplified after 38 cycles (except for *BCAR3α2* in placenta and *BCAR3β* in kidney, which were analyzed after 34 cycles due to artifactual low amplification after 38 cycles) was measured as Integrated Density Values (IDV) using the AlphaImager 2200 program version 5.5 (Alpha Innotech Corporation). Values were normalized to the 400 bp band of the DNA ladder (Invitrogen) to take into account difference between gels and exposure time of the photographs and also to the *G3PDH* bands to take into account the different amounts of cDNA in each template.

2.5. Immunoprecipitation and immunoblotting

Polyclonal rabbit anti-Shep1 SH2 domain antibodies were obtained by using a GST-Shep1 SH2 domain fusion protein as the antigen and for affinity purification; polyclonal rabbit Shep1 C-terminus antibodies were obtained using a peptide corresponding to the last 11 amino acids of Shep1 as the antigen (coupled to BSA) and for affinity purification. Cultured cells were lysed in modified RIPA buffer (1% TritonX-100, 1% Na deoxycholate, 0.1% SDS, 20 mM Tris, 150 mM NaCl, 1 mM EDTA, sodium orthovanadate and protease inhibitors) and tumor tissue was homogenated in phosphate buffer saline, lysed in RIPA buffer, and incubated for immunoprecipitation with anti-Shep1 SH2 antibody or non-immune rabbit IgGs as a control. Samples were separated by SDS-PAGE and probed by immunoblotting with anti-Shep1 C-terminus antibody or with an anti-Cas monoclonal antibody (BD Biosciences) followed by a peroxidase conjugated secondary antibody. Detection was performed by enhanced chemiluminescence detection systems (Amersham Biosciences).

2.6 Immunofluorescence microscopy

Tumors were snap frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound (Sakura Finetek). Five microns cryostat sections were fixed in acetone for 10 min and blocked with 1% BSA, 0.1% Tween20 and 5% normal goat serum in PBS for 30 minutes. The slides were then incubated overnight at 4°C with rabbit anti-Shep1 SH2 antibody (at 5 μg/ml) and rat anti-CD31 antibody (at 1:100, BD Biosciences) followed by a 1 hour incubation with Alexa 488 goat anti-rabbit and Alexa 594 goat anti-rat secondary antibodies (Molecular Probes).

3. Results

3.1. The SHEP1 gene encodes four isoforms with different amino termini

Only one human *SHEP1* splice variant has been reported in the literature, named here *β* (Lu et al., 1999). However, the Ensembl website contains three additional isoforms with different amino-terminal sequences (named here *SHEP1α*, *SHEP1γ*, and *SHEP1δ*) (Figs. 1 and 2;Table 1). The *α* and *β* splice variants were already known in the mouse (Dodelet et al., 1999;Sakakibara and Hattori, 2000;Sakakibara et al., 2003), while we also found the *γ*but not

the δsplice variant in the mouse EST database (Table 1). Reverse-transcription polymerase chain reaction (RT-PCR) amplification of adult mouse brain mRNA confirmed the existence of the entire open reading frame of *Shep1γ* (data not shown). Therefore, the *SHEP1* gene encodes at least three splice variants that are highly conserved in both human and mouse.

Human *SHEP1* spans over 41 kb and contains 15 exons (Fig. 2). Exons 1 and 2 are unique to *SHEP1α* and exon 1 contains the initial ATG for this splice variant (Figs. 1 and 2). Exon 3 contains the initial ATG for *SHEP1β*, exon 4 contains the initial ATG for SHEP1δ, exon 5 is present in splice variants*α*, *β* and δ, but not*γ*, and exon 6 contains the initial ATG for *SHEP1γ*. Therefore, the splice variants differ in their 5′ untranslated regions and amino-terminal sequences but have in common exons 7 to 15.

3.2. The BCAR3 gene generates three splice variants encoding the same isoform and a fourth encoding a shorter isoform

The human *BCAR3* transcript reported in the literature (van Agthoven et al., 1998) is named here *BCAR3α1*. The Ensembl website reports three unpublished *BCAR3* cDNAs containing different 5′ untranslated regions. *BCAR3α2* and *BCAR3α3* encode the same protein as *BCAR3α1*, while *BCAR3β* encodes a shorter protein (Figs. 1 and 2; Table 1). The human *BCAR3* gene spans over 285 kb and contains 17 exons (Fig. 2). Exons 1, 2, and 3 generate the 5′ untranslated region of the *α1* splice variant, exon 4 generates the 5′ untranslated region of the *α2* splice variant, and exon 5 generates the 5′ untranslated region of the *α3* splice variant. Therefore, the three *BCAR3α* transcripts result from alternative splicing of noncoding exons to the common exon 6, which contains the initial ATG for the α variant (Figs. 1, 2). The initial ATG for the *β* splice variant is in exon 8, which also yields the 5′ untranslated region for this variant. Thus, *BCAR3α* and *BCAR3β* have different amino termini. Exons 9 to 17 are common to all four transcripts.

Searches for mouse splice variants similar to the human variants identified only the already published *α* splice variant (Cai et al., 1999) and the *β* variant. RT-PCR amplification of adult mouse brain mRNA confirmed the existence of the entire *Bcar3β*open reading frame (not shown). Exons corresponding to human exons 1 through 5 were not found in mouse through homology searches of NCBI databases. Therefore, mouse *Bcar3* appears to contain only 13 exons generating one *α* and one *β* splice variants.

3.3. NSP1 is expressed in human but not mouse

Human *NSP1* (Lu et al., 1999) contains 10 exons spanning 15 kb (Fig. 2; Table 1). *Nsp1* is also present in the mouse genome with a location corresponding to that in the human genome. However, the mouse *Nsp1* coding sequence contains shifts in the reading frame, stop codons, and an inversion involving exons 2 and 3. In addition, BLAST searches did not reveal any mouse EST sequences. Thus, it appears that the mouse gene cannot generate a full-length Nsp1 protein.

3.4. Tissue expression of SHEP1, BCAR3 and NSP1 splice variants

PCR amplification of cDNAs from adult human tissues showed that the*α*, *β*and *γ* human *SHEP1* splice variants are widely expressed, with somewhat variable levels in different tissues (Fig. 3). We did not detect the expected band for the *δ* splice variant, which should have been co-amplified with *β* as a larger size product (Suppl. Table S1 and data not shown). This is in agreement with the identification of only few ESTs for *δ*. In the mouse, we detected substantial levels of the *α* variant only in the spleen and lung (Suppl. Fig. 1). The *β* and *γ* variants were detected in all tissues and throughout mouse development. The *BCAR3 α1*, *α2* and *β* splice variants were amplified at substantial levels from most tissues, whereas *BCAR3α3* was

amplified at low levels (Fig. 3). Human *NSP1* was detected predominantly in placenta, kidney and pancreas and was not amplified from brain and skeletal muscle (Fig. 3). In contrast, mouse *Nsp1* could not be amplified with primers designed based on the putative mouse exons, consistent with the idea that this gene may not be expressed.

3.5. Expression of SHEP1, BCAR3 and NSP1 in breast cancer cells and endothelial cells

Given the involvement of BCAR3 in estrogen resistance of breast cancer cells (van Agthoven et al., 1998) and the involvement of SHEP1 and NSP1 in JNK activation (Lu et al., 1999;Sakakibara and Hattori, 2000;Sakakibara et al., 2003), we examined mRNA expression patterns for the three genes in several breast cancer cell lines. The cell lines included estrogen-dependent (MCF7 and T-47D) and estrogen-independent (MDA-MB-231, MDA-MB-435 and BT20) breast cancer cells. The non-transformed MCF-10A mammary epithelial cell line and 293T human embryonal kidney cells were also examined for comparison. Substantial levels of *BCAR3* and *NSP1* amplification products were detected in all the cell lines, while *SHEP1* products were low or undetectable (Fig. 4A). Interestingly, however, SHEP1 was amplified at high levels from human umbilical vein endothelial (HUVE) cells. We also detected SHEP1 protein in several endothelial cell lines, but not in the breast cancer cell lines (Fig. 4B and data not shown). These data suggest that a possible role for SHEP1 in cancer progression might involve regulation of tumor angiogenesis and/or tumor blood vessel function.

To investigate whether SHEP1 may play a role in tumor angiogenesis, we examined the expression pattern of SHEP1 in two different mammary tumor models: human MDA-MB-231 tumor xenografts grown in nude mice and mammary tumors from NDL2-5;VEGF transgenic mice. The NL2-5;VEGF tumors overexpress vascular endothelial growth factor (VEGF), which promotes increased tumor vascularization, in addition to a deletion mutant of the ErbB2 oncogene with tumorigenic activity (NDL2-5) (Siegel et al., 1999;Oshima et al., 2004). To determine whether SHEP1 was expressed in the human tumor cells and/or in mouse-derived stromal cells, mRNA from the MDA-MB-231 tumors was amplified using primers common to both human and mouse *SHEP1*. The RT-PCR products were then digested with restriction enzymes allowing distinction between the human and mouse products. We detected only mouse *Shep1* in these tumors (Fig. 5A), even though the mouse stromal cells—including vascular cells, fibroblasts, and blood cells—represent a minor proportion of the tumor mass. This is consistent with the undetectable expression of *SHEP1* in cultured human breast cancer cells and its higher expression in endothelial cells (Fig. 4). Amplification of the three *Shep1* splice variants revealed that they are all present in the stromal cells of these tumors, although the α variant (known to be expressed in blood cells (Lu et al., 1999;Sakakibara and Hattori, 2000)) appears low and was not detected in all tumor samples (Fig. 5A). The presence of the β and γ variants suggests that in these tumors, *Shep1* is highly expressed in the vascular cells and/or other stromal cells, which are derived from the mouse host.

We detected Shep1 protein at higher levels in mammary tumors from NDL2-5;VEGF transgenic mice than in NDL2-5 tumors (Fig. 5B), consistent with the higher blood vessel density in these tumors (Fig. 5C, CD31 staining). Immunofluorescence microscopy confirmed that Shep1 was expressed in tumor blood vessels in both the NDL2-5 and the NDL2-5;VEGF tumors, where Shep1 immunoreactivity was observed in a subset of the CD31-positive endothelial cells (Fig. 5C). Interestingly, in the NDL2-5;VEGF tumors Shep1 appeared to be expressed at higher levels in the blood vessels and also in the tumor cells (Fig. 5C).

In contrast to *SHEP1*, only human *BCAR3* was amplified by RT-PCR from MDA-MB-231 tumor xenografts (Fig. 6A). This indicates that *BCAR3* is present in the tumor cells but not highly expressed in the vasculature of these tumors. Interestingly, *BCAR3α2* was the predominant splice variant amplified from the tumors, whereas it was the least abundant of the

splice variants in MDA-MB-231 cells grown in culture (Fig. 6B). In contrast to the predominance of *BCAR3α2* among the splice variants from MDA-MB-231 tumor tissue, multiple splice variants were amplified at similar levels from most normal tissues (Figs. 3 and 6B). The higher expression of SHEP1 in endothelial cells and tumor stromal cells and of BCAR3 in mammary tumor cells suggest distinctive functions of the two proteins in breast cancer.

4. Discussion

We report that *SHEP1*, *BCAR3* and *NSP1* represent a family of genes including multiple splice variants. The different *SHEP1* and *BCAR3* variants result from alternative splicing events that generate distinct 5′ untranslated regions and amino termini, with the exception of the three *BCAR3* variants encoding the same *α* isoform. Because the multiple *SHEP1* and *BCAR3* variants appear to have different 5′ exons, their transcription is likely controlled by different promoters (Landry et al., 2003). The most 5′ exons are separated by introns large enough to contain a promoter, with the possible exception of the 5′ exons of *BCAR3α2* and *α3*. Alternative promoters can provide a mechanism for regulating expression through different combinations of transcription factors (Landry et al., 2003). This may enable differential regulation, perhaps accounting for the different pattern of *BCAR3* splice variants that we have observed in transformed MDA-MB-231 breast cancer cells grown in culture compared to tumor xenografts.

Although we found most human *SHEP1* and *BCAR3* splice variants to be widely expressed, mouse *Shep1α* was amplified at substantial levels only from the spleen and the lung, in agreement with previous data at the mRNA and protein level (Lu et al., 1999;Sakakibara and Hattori, 2000). In contrast, we detected human *SHEP1α* in all tissues. It is not known why the expression patterns of the human and mouse *SHEP1α* splice variants may be different and whether there are corresponding differences in protein expression.

There is evidence that *SHEP1* and *BCAR3* may be expressed in different cell types from the same tissue. For example, we found that SHEP1 is preferentially expressed in cultured endothelial cells and tumor stromal cells. Whether SHEP1 expression in endothelial cells and/or blood cells may substantially account for the expression observed in some tissues remains to be determined. Furthermore, in the mouse brain *Shep1* mRNA is detected by *in situ* hybridization in the pyramidal neurons of the hippocampus while *Bcar3* is detected in cerebellar Purkinje neurons (Allen Brain Atlas, www.brain-map.org). The splice variants present in each cell type, however, remain to be characterized. We found a more restricted distribution for *NSP1* mRNA in tissues, consistent with previous reports (Lu et al., 1999), but widespread expression in the human cell lines examined. The factors regulating *SHEP*1, *BCAR3* and *NSP1* mRNA expression have not been characterized, except that proinflammatory cytokines have been shown to upregulate *BCAR3* expression (Cai et al., 1999).

Certain *SHEP1* (*γ*and *δ*) and *BCAR3* (*α1*, *α2* and *α3*) splice variants and *NSP1* contain ATG sequences that could initiate translation upstream of the initial ATG due to favorable Kozak sequences (Fig. 1 and accession number AF124249 for *NSP1*) (Kozak, 2002). This likely decreases the translation efficiency of the main open reading frame (Kozak, 2002). In addition, the initial ATG of *NSP1* is in an unfavorable sequence context for translation initiation (Kozak, 2002). Furthermore, the high (>70%) GC content of the 5′ untranslated regions of *SHEP1*β, *SHEP1*γ, *BCAR3α2* and *BCAR3α3* (Fig. 1) suggest the presence of secondary structure. High levels of helicase activity from the eIF4F translation initiation complex may therefore be required for protein synthesis from these variants (Gingras et al., 2001;Kozak, 2002). The upstream ATGs and high 5′ GC content suggest that translation of most *SHEP1*, *BCAR3* and *NSP1* variants is tightly regulated, as is typical of genes encoding proteins whose

overproduction could disrupt tissue homeostasis and cause disease (Gingras et al., 2001;Kozak, 2002). Synthesis of splice variants with high GC content in their 5′ untranslated regions is expected to be low in normal tissues but upregulated in many tumors because of deregulated eIF4F helicase activity.

Consistent with a role in tumorigenesis, many *SHEP1*, *BCAR3* and *NSP1* ESTs are derived from a variety of cancer cells and microarray analyses have also detected transcripts in cancer cells and tumor specimens (http://symatlas.gnf.org and http://source.stanford.edu), but it remains to be conclusively shown whether the expression of these genes is upregulated in tumors compared to normal tissues. Interestingly our immunohistochemical data suggest that VEGF, a growth factor known to contribute to tumor progression, promotes Shep1 expression because we observed higher Shep1 expression in the endothelial cells and also in the tumor cells of NDL2-5;VEGF tumors compared to NDL2-5 tumors. However, the mechanism for the effects of VEGF on SHEP1 expression will need to be further investigated.

The known activities of SHEP1, BCAR3 and NSP1 support the idea that these proteins could play a role in cancer. For example, overexpression of BCAR3 in breast cancer cells has been reported to induce antiestrogen resistance, indicating a role in promoting cell cycle progression and sustaining cell proliferation (van Agthoven et al., 1998;Cai et al., 2003b). SHEP1, BCAR3 and NSP1 could also play a role in invasion and metastasis because they have been shown to regulate cytoskeletal dynamics, cell adhesion and migration, and JNK activation (Sakakibara et al., 2002;Cai et al., 2003a;Cai et al., 2003b;Riggins et al., 2003;Dail et al., 2004).

Our data suggest that SHEP1 is highy expressed in tumor blood vessels, where it could have a role in promoting tumor angiogenesis downstream of both integrins and tyrosine kinase receptors for angiogenic growth factors (Yancopoulos et al., 2000). Additional studies are needed to further elucidate the role of *SHEP1*, *BCAR3* and *NSP1* in cell transformation and tumor angiogenesis. A better knowledge of the differential expression of these genes will help to understand how they contribute to tumorigenesis and the development of antiestrogen resistance in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCAR3
: breast cancer antiestrogen resistance locus 3

NSP1
: novel SH2-containing protein 1

PCR
: polymerase chain reaction

SHEP1
: SH2 domain-containing Eph receptor-binding protein 1

RT-PCR
: reverse-transcription PCR

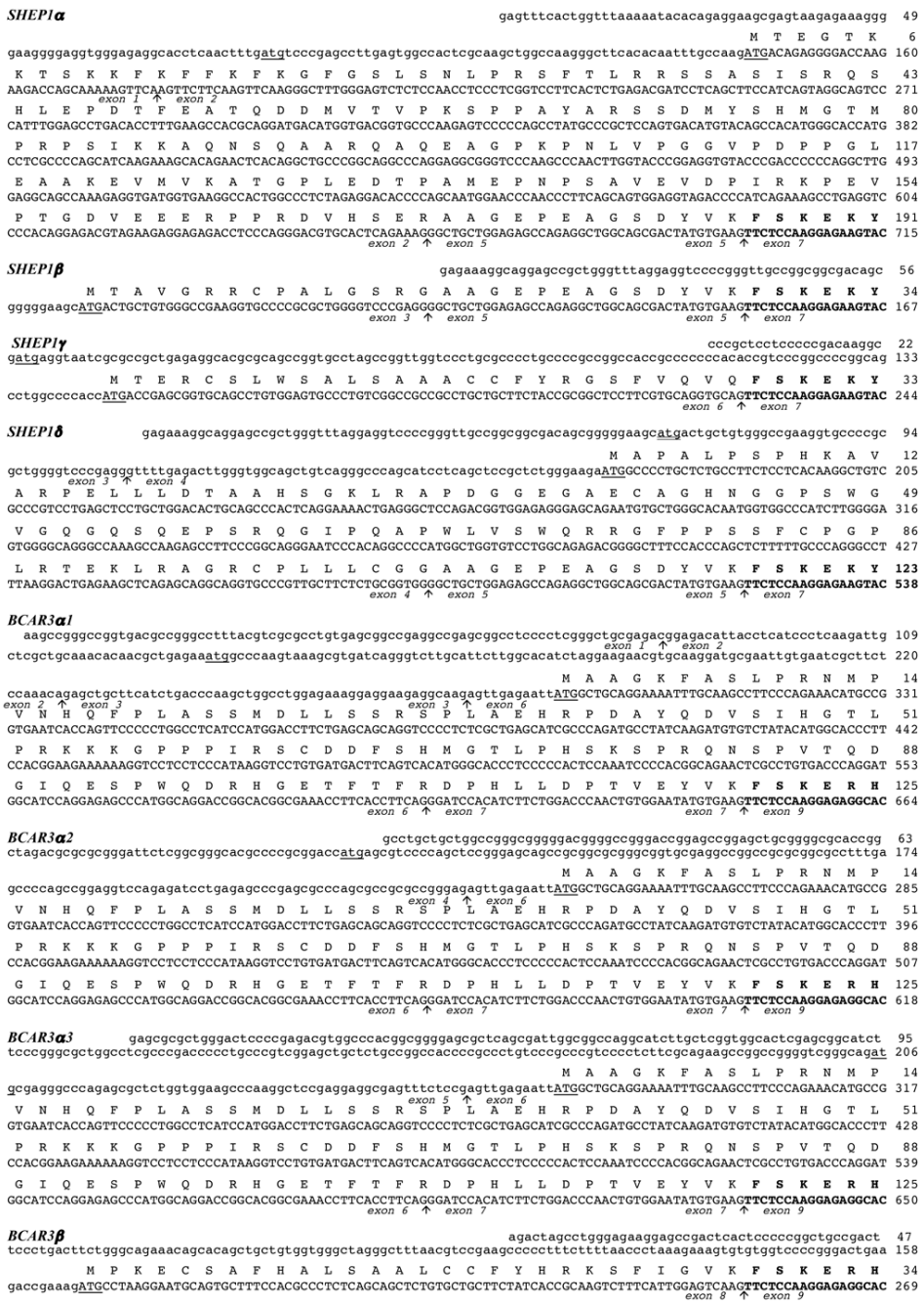

Fig 1.

SHEP1 and *BCAR3* splice variants. The 5′ untranslated regions (lowercase letters) and the sequences encoding the amino-terminal portions (uppercase letters) of the four *SHEP1* splice variants and the four *BCAR3* splice variants are shown next to the deduced amino acid sequences. The first exon common to the *SHEP1* splice variants (exon 7) and to the *BCAR3* splice variants (exon 9) is depicted in bold. Arrows indicate the junctions between exons. The translation start site of the main open reading frames and the ATG sequences in the 5′ untranslated regions are underlined; the atg with a double underline in *SHEP1δ* correspond to the initial ATG in *SHEP1β* but is followed by a stop codon in this isoform. Accession numbers corresponding to the sequences shown are listed in Table 1.

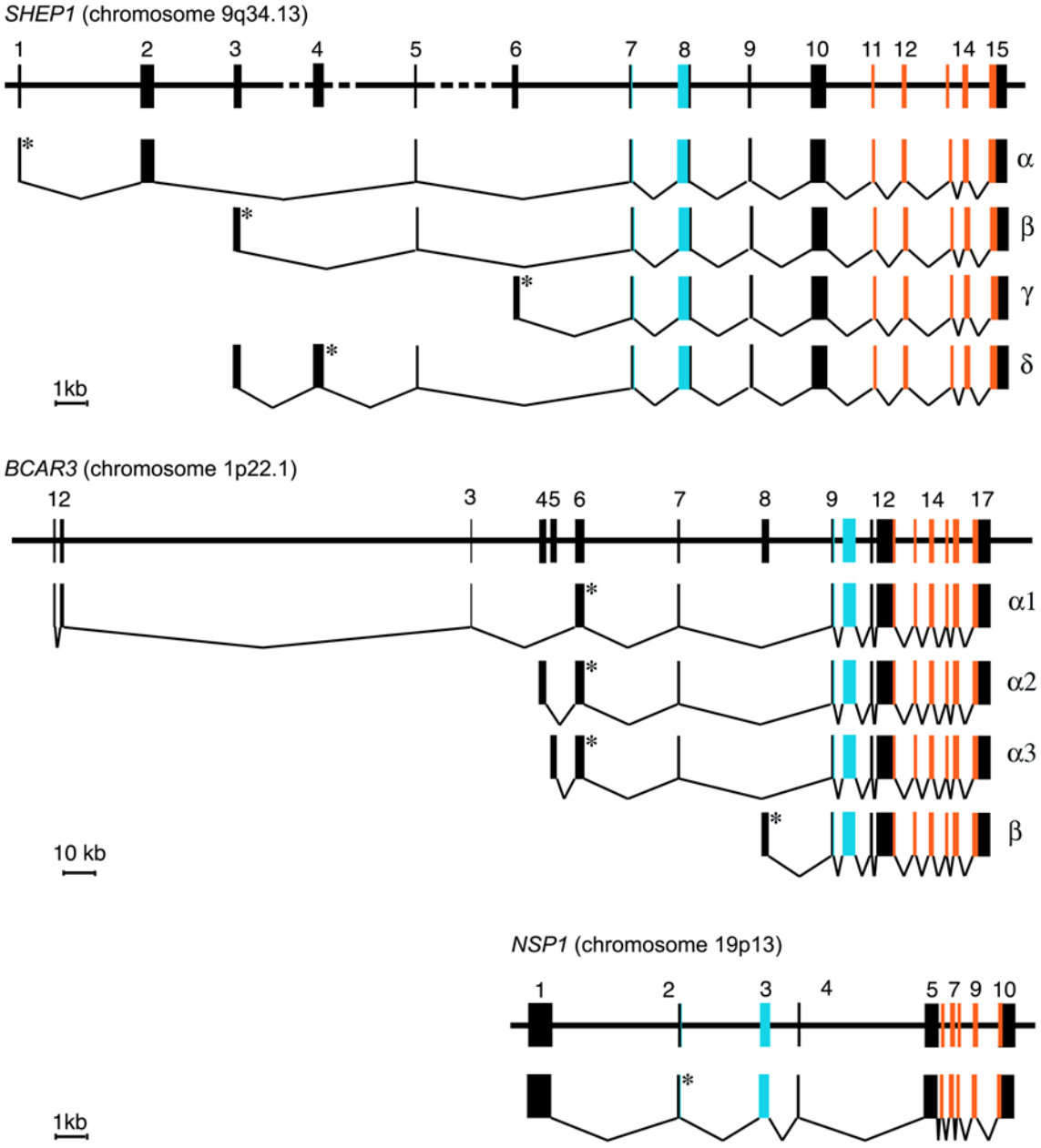

Fig 2.

Genomic organization and alternatively spliced forms of human *SHEP1*, *BCAR3* and *NSP1*. Exons are depicted as boxes and numbered from the 5′ end. Blue exons encode the SH2 domain and orange exons encode the exchange factor-like domain. Sizes are to scale, except for *SHEP1* introns 3 and 4 and 5. Translation start sites are indicated by an asterisk.

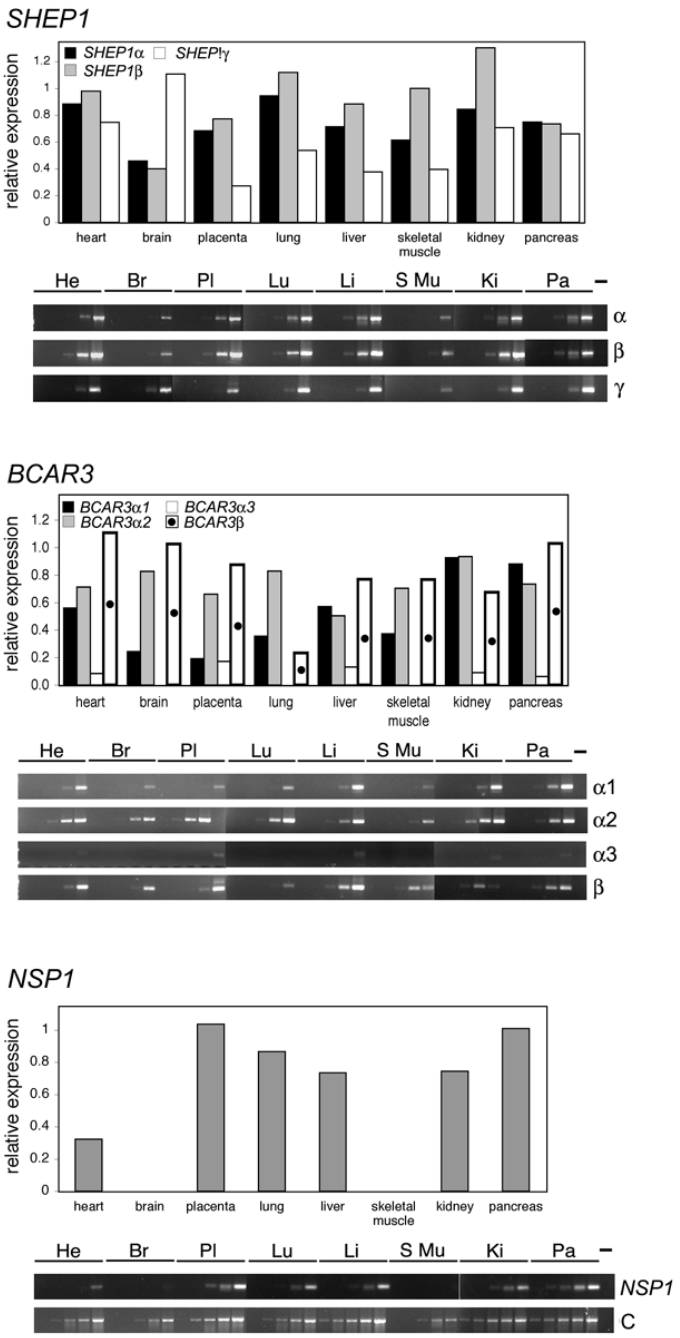

Fig 3.

Expression profiles of *SHEP1*, *BCAR3*, and *NSP1* splice variants in adult human tissues. cDNAs from various tissues were screened by PCR amplification. For each tissue, the PCR products were analyzed at 22, 26, 30, 34 and 38 cycles on an agarose gel. The histograms show quantification of the bands as described in the Materials and Methods. C, *G3PDH* control amplification. "–", control with no cDNA in the PCR reaction; He, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; S Mu, skeletal muscle; Ki, kidney; and Pa, pancreas.

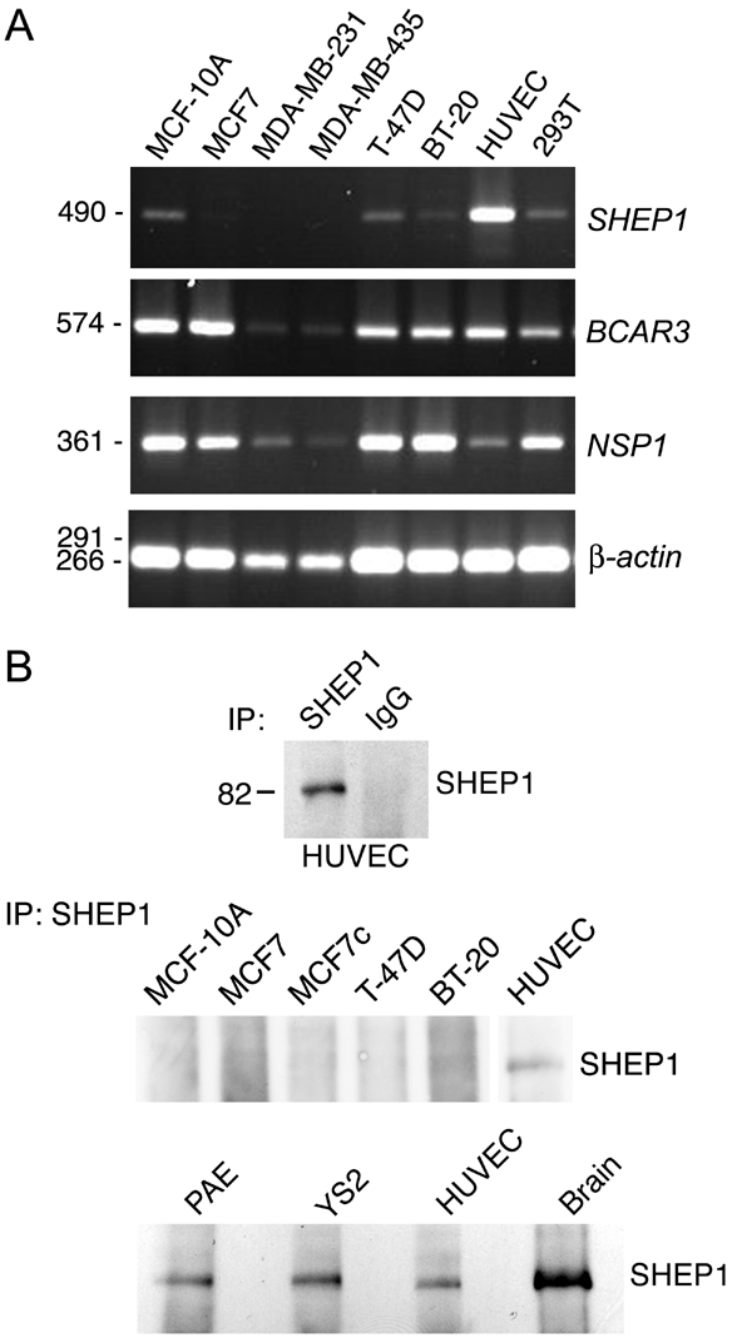

Fig 4.

Expression profiles of *SHEP1*, *BCAR3*, and *NSP1* in different human cell types. (A) Non-transformed MCF-10A mammary epithelial cells, MCF7 and T-47D, MDA-MB-231, MDA-MB-435 and BT20 breast cancer cells, endothelial HUVE cells, and embryonal kidney 293T cells were screened by RT-PCR using 35 cycles of amplification. β-actin was amplified as a reference. The *SHEP1* and *BCAR3* primers were designed to amplify all the splice variants. (B) SHEP1 was immunoprecipitated (IP) from some of the cell lines shown in (A) as well as porcine aortic endothelial cells (PAE) and mouse yolk sac endothelial cells (YS2), and brain as a positive control. The immunoprecipitates were probed with anti-SHEP1 antibodies. Immunoprecipitates with non-immunized rabbit IgGs served as negative controls.

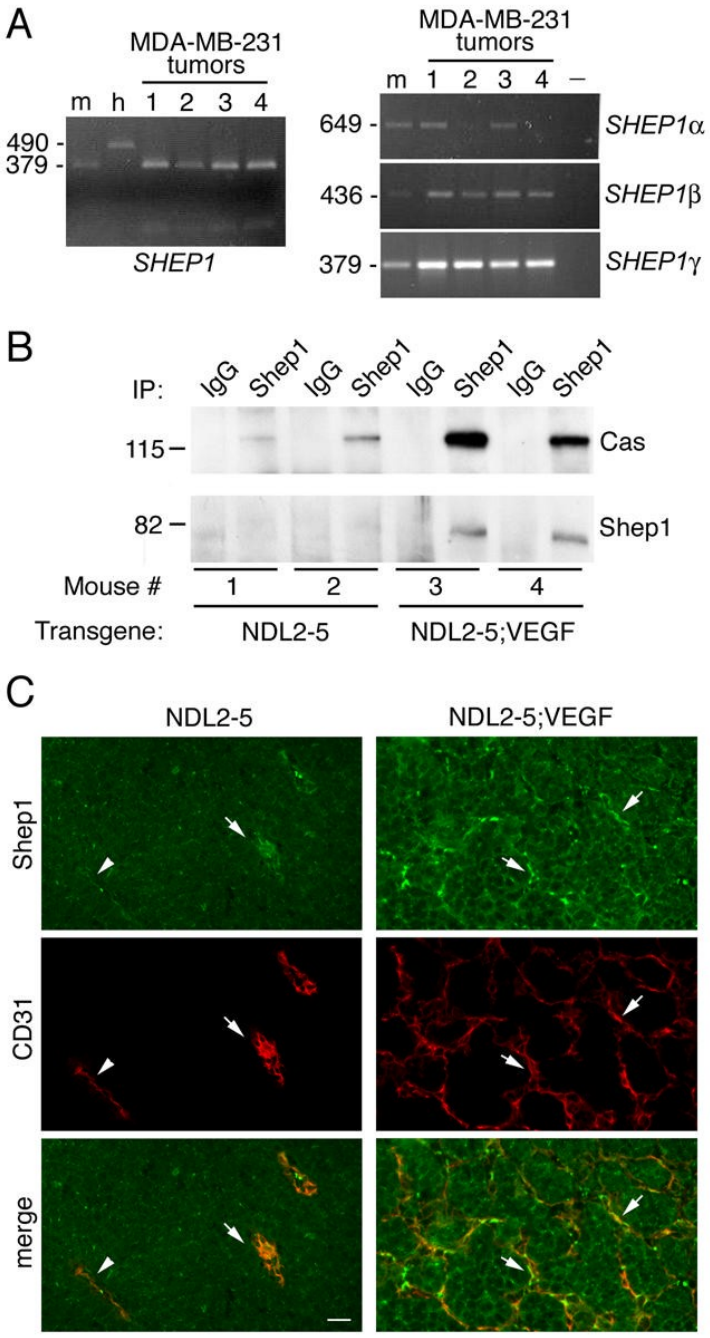

Fig 5.

SHEP1 is expressed in endothelial cells in mouse mammary tumor models. (A) Four MDA-MB-231 tumor xenografts were screened by RT-PCR with *SHEP1* primers corresponding to sequences conserved in human and mouse using 35 cycles of amplification. Digestion of the *SHEP1* amplification products with a restriction enzyme that only digests the mouse sequence shows that in the tumors *SHEP1* is predominantly in the mouse cells (left panel). Primers specific for each splice variant were also used for amplification (right panel). m, control amplification from mouse lung cDNA (for *SHEP1α*) and brain cDNA (for *SHEP1*, *SHEP1β*, and *SHEP1γ*); h, control amplification from human control cDNA (BD Biosciences Clontech); "–", control with no cDNA in the PCR reaction. (B) Shep1 was immunoprecipitated (IP) from

mouse mammary tumors overexpressing a deletion mutant form of the ErbB2 oncogene alone (NDL2-5) or together with VEGF (NDL2-5;VEGF), which greatly increases tumor vascularization. Antibodies from a non-immunized rabbit (IgG) were used in the immunoprecipitations as a negative control. The immunoprecipitates were probed with antibodies to Shep1 and also to Cas, which co-immunoprecipitates with Shep1 and can be detected with higher sensitivity. Shep1 expression is low in NDL2-5 tumors but substantial in NDL2-5;VEGF tumors. (C) Frozen sections from NDL2-5 and NDL2-5;VEGF tumors were double-labeled with anti-Shep1 antibodies and with antibodies to the endothelial cell marker CD31. Shep1 immunoreactivity is evident in a subset of endothelial cells in both tumors (arrows). Arrowheads mark a blood vessel that does not express detectable Shep1. Scale bar, 20 μm.

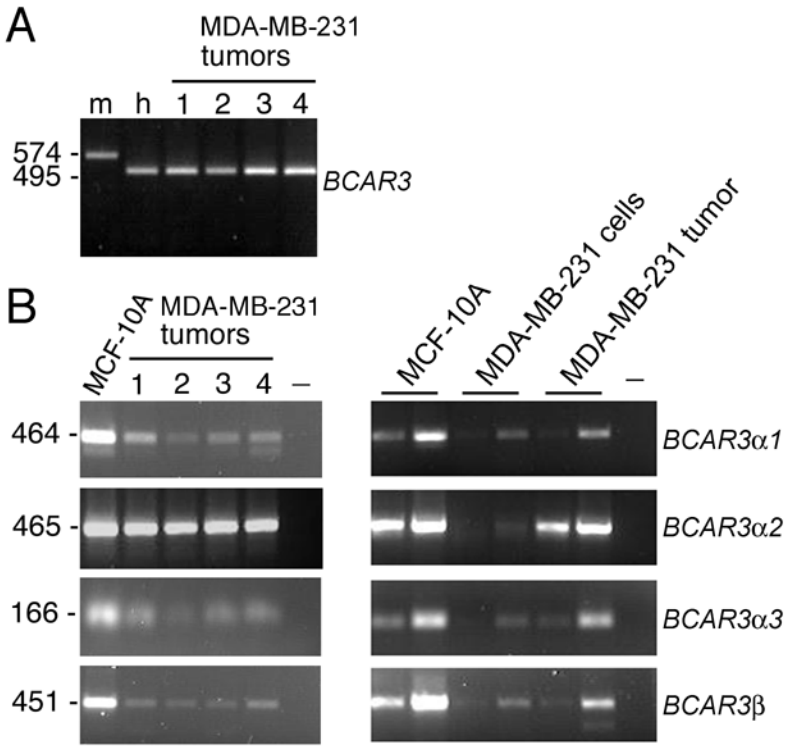

Fig 6.

BCAR3 is expressed in tumor cells in mouse xenografts grown from MDA-MB-231 breast cancer cells. (A) Four MDA-MB-231 tumor xenografts were screened by RT-PCR with *BCAR3* primers corresponding to sequences conserved in human and mouse using 35 cycles of amplification. Digestion of *BCAR3* amplified products with a restriction enzyme that only digests the human sequence shows that, in the tumors, the *BCAR3* PCR product is predominantly in the human cells. (B) Amplification with primers specific for each of the four *BCAR3* splice variants shows that they are all present, but *α2* is the most readily amplified among them (left panel). Amplification of the *BCAR3* transcripts shows the preferential amplification of *α2* relative to the other splice variants in MDA-MB-231 tumor tissue, whereas all splice variants are amplified at similar levels in cultured MDA-MB-231 and MCF-10A cells. Amplifications were carried out for 38 cycles (left panel) or 34 and 38 cycles (right panel).

Table 1

Accession numbers for the *SHEP1*, *BCAR3* and *NSP1* splice variants

Splice variant	Amino acids in the encoded isoform	Human	Mouse
SHEP1α	860	BC032365(DB161948)[a]	AF168364
SHEP1β	703	AF124251(BI912337)[a]	AB030442
SHEP1γ	702	AK056068	AK172072
SHEP1δ	792	BX647905	-
BCAR3α_1	825	BC028477	-
BCAR3α_2	825	AF124250	AF179566
BCAR3α_3	825	AK055442	-
BCAR3β	734	AL833121	CF909407[b]
NSP1	576	AF124249	-

[a] Accession number of an EST containing the entire 5′ untranslated sequences shown in Fig. 1.

[b] Accession number of an EST containing the entire 5′ untranslated sequences and 5′ coding sequence shown in Fig. 1. The databases do not contain a full length mRNA sequence for this form.